Expression of GnRH receptor in the canine corpus luteum, and luteal function following deslorelin acetate-induced puberty delay

Kaya, D ; Gram, A ; Kowalewski, Mariusz P ; Schäfer-Somi, S ; Kuru, M ; Boos, Alois ; Aslan, S

Abstract: The goals of this study were as follows: (Experiment 1) to examine the basic capability of canine corpora lutea (CL) to respond to GnRH by assessing expression of gonadotropin-releasing hormone receptor (GnRH-R) in luteal samples collected throughout the luteal lifespan from non-pregnant dogs, and (Experiment 2) to investigate the effects of pre-pubertal application of the GnRH agonist deslorelin acetate on luteal function following the first oestrus. Mature CL were collected during the mid-luteal phase (days 30–45) from treated and control bitches. Transcript levels of several factors were determined: estrogen receptors (ESR1/ER , ESR2/ER), progesterone (P4)-receptor (PGR), prolactin receptor (PRLR), PGE2-synthase (PTGES) and PGE2 receptors (PTGER2/EP2, PTGER4/EP4), vascular endothelial growth factor (VEGFA) and VEGF receptors (VEGFR1 and VEGFR2), cyclooxygenase 2 (COX2/PTGS2), steroidogenic acute regulatory protein (STAR) and 3 -hydroxysteroid dehydrogenase (3 HSD). Additionally, levels of Kisspeptin 1 (Kiss1) and its receptor (KISS1-R) were evaluated. Although generally low, GnRH-R expression was time dependent and was elevated during early dioestrus, with a significant decrease towards luteal regression. In deslorelin-treated and control dogs, its expression was either low or frequently below the detection limit. EP2 and VEGFR1 were higher in the treated group, which could be caused by a feedback mechanism after long-term suppression of reproductive activity. Despite large individual variations, 3 HSD was higher in the deslorelin-treated group. This, along with unchanged STAR expression, was apparently not mirrored in increased luteal functionality, because similar P4 levels were detected in both groups. Finally, the deslorelin-mediated long-term delay of puberty does not have negative carry-over effects on subsequent ovarian functionality in bitches.

DOI: https://doi.org/10.1111/rda.13038

Originally published at:
Kaya, D; Gram, A; Kowalewski, Mariusz P; Schäfer-Somi, S; Kuru, M; Boos, Alois; Aslan, S (2017). Expression of GnRH receptor in the canine corpus luteum, and luteal function following deslorelin acetate-induced puberty delay. Reproduction in Domestic Animals, 52(6):1104-1112.
DOI: https://doi.org/10.1111/rda.13038

Expression of GnRH receptor in the canine corpus luteum, and luteal function following deslorelin acetate-induced puberty delay

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Short title: Canine luteal function after GnRH-agonist treatment

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Contents

The goals of the present study were: (Experiment 1) to examine the basic capability of canine corpora lutea (CL) to respond to GnRH by assessing expression of gonadotropin releasing hormone receptor (*GnRH-R*) in luteal samples collected throughout the luteal lifespan from non-pregnant dogs, and (Experiment 2) to investigate the effects of pre-pubertal application of the GnRH agonist deslorelin acetate on luteal function following the first estrus. Mature CL were collected during the mid-luteal phase (days 30-45) from treated and control bitches. Transcript levels of several factors were determined: estrogen receptors (*ESR1*/*ERα*, *ESR2*/*ERβ)*, progesterone (P4)-receptor (*PGR*), prolactin receptor (*PRLR*), PGE2-synthase (*PTGES*) and PGE2 receptors (*PTGER2*/*EP2*, *PTGER4*/*EP4*), vascular endothelial growth factor (*VEGFA*) and VEGF receptors (*VEGFR-1* and *-2*), cyclooxygenase 2 (*COX2*/*PTGS2*), steroidogenic acute regulatory protein (*STAR*) and 3β-hydroxysteroid dehydrogenase (*3βHSD*). Additionally, levels of Kisspeptin 1 (*Kiss1*) and its receptor (*KISS1-R*) were evaluated. Although generally low, *GnRH-R* expression was time-dependent and was elevated during early diestrus, with a significant decrease towards luteal regression. In deslorelin-treated and control dogs, its expression was either low or frequently below the detection limit. *EP2* and *VEGFR-1* were higher in the treated group, which could be caused by a feedback mechanism after long-term suppression of reproductive activity. Despite large individual variations, *3βHSD* was higher in the deslorelin-treated group. This, along with unchanged *STAR* expression, was apparently not mirrored in increased luteal functionality, because similar P4 levels were detected in both groups. Finally, the deslorelin-mediated long-term delay of puberty does not have negative carry-over effects on subsequent ovarian functionality in bitches.

Key words: deslorelin, domestic dog (Canis familiaris), GnRH receptor, luteal function, puberty delay

Introduction

Gonadotropin releasing hormone (GnRH) is a key regulator of sexual maturation and reproductive function in mammals that may also modulate the activity of diverse systems in the brain and many peripheral organs (Ramakrishnappa et al. 2005). The GnRH receptor (GnRH-R), also known as luteinizing hormone-releasing hormone receptor (LHRHR), is a member of the G protein-coupled receptors family. When activated by the hypothalamic GnRH, GnRH-R undergoes a conformational change, stimulates a G-protein and leads to the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are responsible for the regulation of ovarian and testicular functions (Sealfon et al. 1997, Stojilkovic and Catt 1995, Cheung and Wong 2008).

The secretion of GnRH is regulated by Kisspeptin (Kiss1) (Seminara et al. 2003, Funes et al. 2003). By acting through its G-protein coupled receptor, Kiss1-R, Kiss1 centrally stimulates pulsatile GnRH release (Seminara et al. 2003, Funes et al. 2003). Kiss1 and its receptor were also found in human and rat ovarian tissues, as well as in granulosa-lutein cells *in vitro* (Gaytan et al. 2009, Cejudo Roman et al. 2012). Also the presence of *GnRH-R* transcripts was confirmed in ovarian follicles and corpus luteum (CL), *e.g.*, in mouse (Torrealday et al. 2013) and human (Choi et al. 2006). Thus, functional interactions between these two systems, *i.e.*, GnRH and Kiss, within the ovary have been implicated.

Long-term release GnRH analogs are synthetic compounds that interact and stimulate GnRH-R. After an initial sudden increase (i.e. flare-up) effect upon binding to the receptor, the sustained action of GnRH or its agonists (GnRH-a) causes GnRH-R down-regulation. The inhibitory actions of GnRH and/or GnRH-a on gonadal steroidogenesis involve suppression of gonadotropin receptors or intermediary enzymes involved in steroidogenic pathways in the ovary, *e.g.*, P450scc enzyme, 3β-hydroxysteroid dehydrogenase (3βHSD) or aromatase (Khosravi and Leung 2003, Janssens et al. 2000, Clayton et al. 1979, Sridaran et al. 1999b). In some reports, GnRH-a were suggested to suppress expression of FSH and LH receptors (Tilly et al. 1992, Guerrero et al. 1993), and to decrease gonadotropin-induced cAMP levels or the activities of steroidogenic factors such as steroidogenic acute regulatory (STAR) protein (Sridaran et al. 1999b, Shinohara et al. 1985). The presence of both GnRH and the GnRH-R in the extrapituitary tissues implies an autocrine/paracrine role for GnRH and a potential site of action for GnRH-a (Aguilar-Rojas and Huerta-Reyes 2009).

With regards to the dog, only one GnRH isoform (*GnRH-I*) and one receptor type have been identified in reproductive tissues so far, and their spatial and temporal expression in canine uterus and placenta have been described recently (Schafer-Somi et al. 2015). Similar to other mammals, also in the dog GnRH by acting centrally modulates both inhibitory and stimulatory responses affecting ovarian function and regulating cyclicity (reviewed in (Gobello 2007)). Importantly, however, with respect to canine luteal function, neither LH nor GnRH seem to be mandatory at the early luteal stage (Onclin et al. 2000). Instead, prostaglandins (PGs) produced locally, *i.e.*, within the CL, and in particular PGE2, appear to be the main luteotrophic factors involved in establishment of luteal function (reviewed in (Kowalewski 2014)). Concerning the luteotrophic effect of

PGE2, it was found to induce progesterone (P4) output in lutein cells *in vitro* (Kowalewski et al. 2013). Similarly, interfering with cyclooxygenase 2 (COX2, PTGS2) *in vivo* with its specific inhibitor (firocoxib, Previcox, Merial), decreases STAR and 3βHSD mRNA and protein expression (Kowalewski et al. 2015).

Despite recent progress in understanding canine luteal physiology in pregnant and non-pregnant cycles (Kowalewski 2014, Kowalewski 2012, Kowalewski et al. 2015), the mechanisms of suppression and resumption of ovarian activity and, subsequently, luteal function after short-/long- term inhibition of gonadal activity remains poorly understood. Therefore, the first aim of the present study was to investigate the presence of GnRH-R in CL from non-pregnant dogs (days 5, 15, 25, 35, 45 and 65 post-ovulation, p.o.) during the non-pregnant cycle. With this goal, we aimed to establish the basic capability of canine CL to respond to GnRH. Additionally, the expression of *GnRH-R*, *Kiss1*, *Kiss1-R*, *3βHSD*, *PGR*, *STAR*, *COX2* (*PTGS2*), *PTGES*, *EP2* (*PTGER2*), *EP4* (*PTGER4*) and the *VEGF* system was investigated at mid-luteal phase following the first estrus after long-term pre-pubertal GnRH agonist treatment.

Materials and Methods

Animals, treatments and tissue materials

For Experiment 1, corpora lutea (CL) were collected by routine ovariohysterectomy (OHE) from sexually mature (2-8 years old), healthy, crossbreed, non-pregnant dogs throughout diestrus at days: 5 (n=4), 15 (n=5), 25 (n=5), 35 (n=4), 45 (n=3) and 65 (n=3) post ovulation (p.o.), and were used for assessment of GnRH-R gene expression. Blood plasma P4 concentrations and vaginal cytology were used to determine the cycle stage. P4 levels of at least 5 ng/ml were considered as the day of ovulation (Concannon

et al. 1989). For RNA preservation, tissue samples were placed in RNAlater (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at +4°C and afterwards they were stored at -80 °C until total RNA extraction as described previously (Kowalewski et al. 2006a). For non-radioactive in situ hybridization (ISH) CL were trimmed of surrounding tissues and fixed in 10% neutral phosphate-buffered formalin for 24 h at +4°C.

In experiment 2, the effects of pre-pubertal application of the GnRH agonist deslorelin acetate on canine luteal function following the first estrus were assessed using luteal samples that originated from the same animals as in a previous study (Kaya et al. 2015). Briefly, thirteen healthy, caucasian shepherd and kangal crossbreed, pre-pubertal bitches aged 4.2±0.6 months with a body weight of 9.5±3.4 kg were used. Implants containing either 9.4 mg (n = 5) or 4.7 mg (n = 4) deslorelin (Suprelorin; Virbac, France), or placebo (sodium chloride 0.9%; n = 4), were administered subcutaneously in the inter-scapular region using a single use applicator. The signs of estrus were monitored once daily by assessing physical (vulvar appearance and swelling, sero-sanguinous vaginal discharge) and sexual behavioral changes, until occurrence of the first estrus. Vaginal cytology, serum P4 and estradiol-17β (E2) concentrations were measured every other day. No clinically detectable systemic side effects were observed in any of the treated bitches. Control, and those animals that showed estrus signs were subjected to OHE during the mid-luteal phase (days 30-45) and mature corpora lutea (CL) were collected. The onset of puberty appears quite variable within several small and large breeds of dogs. While many smaller breeds reach puberty between 6 and 10 months of age, in some larger breed bitches onset of the first estrous cycle is seen at 2 years of age (England et al. 2010, Root Kustritz 2010). Accordingly, as reported

144 previously (Kaya et al. 2015), control animals used in our study showed estrus within
145 61.9 ± 9.7 weeks after placebo treatment. Four (n=4) of the deslorelin-treated bitches
146 (n=2 in each of the implanted groups) came into estrus during the 82.7±8.9 weeks of
147 observation. The other treated bitches (n=3 and n=2 in groups treated with 9.4 mg and
148 4.7 mg Suprelorin, respectively) did not show estrus by the end of the observation
149 period at 101.5 weeks when the observation period finished. Consequently, samples
150 from these 4 dogs, which came into estrus, were merged into one group for further
151 analyses including semi-quantitative PCR. For mRNA-analysis, tissues were excised,
152 embedded in Tissue-Tek® (Sanova Pharma GmbH, Vienna, Austria) and frozen at -80°C
153 until further use.
154 All experimental procedures were reviewed and approved by the respective animal
155 welfare authorities of either the Justus Liebig University Giessen, Germany (permit no
156 II 25-3-19c20/15c GI 18/14), or Local Animal Ethics Committee of the Faculty of
157 Veterinary Medicine, Kafkas University, Turkey (KAÜ-HADYEK; 2010/30 and
158 2014/016).
159
160 **RNA isolation and semi-quantitative RT-PCR**
161 From all samples, total RNA was extracted by using TRIzol®-Reagent (Life
162 Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The
163 RNA concentrations were measured with a NanoDrop 2000C spectrophotometer
164 (Thermo Scientific, Wilmington, DE, USA). RNA quality was assessed by checking the
165 RNA integrity numbers (RIN). For elimination of genomic DNA contamination, total
166 RNA was DNAse-treated with RQ1 RNase-free DNase (Promega, Dübendorf,
167 Switzerland) according to the manufacturer's instructions. Reverse transcription of

isolated RNA was performed in an Eppendorf Mastercycler (Vaudaux-Eppendorf AG, Basel, Switzerland) using reagents from Applied Biosystems by Thermo Fisher Scientific (Foster City, CA, USA). Water instead of cDNA and the so-called RT-minus controls were run as negative controls. Then, semi-quantitative (TaqMan) RT-PCR analyses were performed in an automated fluorometer ABI PRISM 7500 Sequence Detection System from Applied Biosystems as previously described, ensuring approximately 100% efficiency of reactions (Kowalewski et al. 2006a, Kowalewski et al. 2011a, Kowalewski et al. 2006b). Reaction conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles consisting of 95 °C for 15s and 60 °C for 60s. Three different reference genes (*GAPDH*, *CYCLOPHILIN A* and *β-ACTIN*) were used for normalization and relative expression changes were calculated using the comparative CT (ΔΔCT) method according to the protocol of the manufacturer of the ABI Prism 7500. The list of primers and TaqMan probes used in the semi-quantitative real time PCR are presented in Table 1. In addition, canine-specific TaqMan Gene Expression Assays purchased from Applied Biosystems were used for *CYCLOPHILIN A* (Prod. No. Cf03986523_gH), *β-ACTIN* (Prod. No. Cf03023880_g1), *GnRH-R* (Prod. No. Cf02623893_m1), and *Kiss1-R* (Prod. No. Cf02715933_u1).

Hormone analysis

Blood samples were centrifuged for 3 min at 3000 g and the sera obtained were stored at -18°C until analysis. The concentrations of P4 (ng/ml) and E2 (pg/ml) were measured in serum samples using the electro-chemiluminescence immunoassay (ECLIA) with a fully automated Cobas Modular E170 Analyzer (Roche Diagnostics, Mannheim,

192 Germany) in an internationally certified commercial laboratory (Düzen Laboratories
193 Group, Ankara, Turkey) as described before (Agaoglu et al. 2011). Extraction efficiency
194 was >95%. The mean intra- and inter-assay coefficients of variance for P4 were 3.2 %
195 and 1.7%, respectively. The analytic sensitivity was 0.03 ng/ml. Values for E2 assay
196 were 5.0 pg/ml (sensitivity), 3.0% (intra-assay coefficient) and 1.7% (inter-assay
197 coefficient).
198
199 ***In situ* hybridization (ISH)**
200 A non-radioactive ISH method was carried out to localize *GnRH-R* mRNA in the canine
201 CL. Total RNA was extracted and hybridization probes were prepared following the
202 same method used in our previous studies (Kowalewski et al. 2006a). Briefly, reverse
203 transcription was performed using the following canine specific primers in order to
204 prepare (cRNA) probes: *GnRH-R* forward 5`- CGC CTC TCC TGA ACA GAA TC-3`,
205 *GnRH-R*, reverse 5`- GTT GGC CAA GGT CAG ATG TT-3`; the amplicon length was
206 250 bp. The PCR products were separated on ethidium bromide-stained 2% agarose
207 gels, then isolated using the Qiaex II gel extraction system (Qiagen GmbH Hilden,
208 Germany) and subcloned into the pGEM-T vector (Promega, Duebendorf, Switzerland).
209 Incorporation of selected genes in the pGEM-T vector was proved by control-digestion
210 with NcoI and NotI restriction enzymes (New England Biolabs, Frankfurt, Germany)
211 and sent for commercial sequencing (Microsynth, Balgach, Switzerland). Afterwards,
212 pGEM-T vectors were linearized with the restriction enzymes NcoI and NotI, for
213 antisense cRNA and sense cRNA, respectively. Hybridization probes were prepared
214 using the DIG-RNA labelling kit according to the manufacturer's protocols (Roche
215 Diagnostics, Mannheim, Germany) Then, cRNA probes were applied to each section

and incubated overnight at 37°C for hybridization. Afterwards, hybridized probes were detected with alkaline phosphatase-conjugated, sheep anti-DIG Fab Fragments (Roche Diagnostics) diluted 1:5000, and signals were visualized using the substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics). No counterstaining was applied.

Statistical analysis

GraphPad version 3.06 software (GraphPad Software, Inc., San Diego, California, USA) was used to assess differences in target gene expression. Statistical analysis was done using either the Kruskal Wallis test (a nonparametric ANOVA) followed by Dunn's multiple comparison test to examine the effect of time on *GnRH-R* expression in canine CL throughout the luteal life span, or else an unpaired two-tailed Student's t-test was applied for pairwise comparisons. Values of $P<0.05$ were considered significant. In experiments evaluating gene expression following deslorelin treatment, the numerical data are presented as geometric means with geometric standard deviation (Xg ± SD). Mean values ± standard deviation (SD) are presented for experiments evaluating *GnRH-R* expression throughout diestrus. For the analyses of P4 and E2 concentrations, PASW statistics software (version 17 for Windows, SPSS Ltd, Hong Kong) was used. Normality and homogeneity of groups were determined by the Shapiro-Wilk test. Due to the relatively small sample numbers, a non-parametric Mann-Whitney U test was performed for comparisons between groups (*i.e.*, treated *vs*. control). Data were summarized as descriptive statistics, display concentrations of the respective hormone on the day of tissue sample collection and are shown as box and whisker plots (Fig. 1).

240 **Results**

241 **Hormonal profiles**

242 No significant differences were observed for P4 and E2 concentrations between treated
243 and control animals at the time of tissue collection (P>0.05, Fig. 1).

244

245 **Localization of canine *GnRH-R* mRNA and its expression in canine CL**
246 **throughout the luteal phase in non-pregnant dogs.**

247 Although generally low, *GnRH-R* expression was time-dependent in non-pregnant dogs
248 CL throughout the luteal phase (P<0.0001). The highest expression was detected at the
249 beginning of the luteal phase, at days 5 and 15 p.o.. This was followed by a significant
250 decrease towards the late luteal phase (days 5 *vs*. 65 p.o. P<0.001, and days 15 *vs*. 65
251 p.o. P<0.01) (Fig. 2A).

252 In order to localize *GnRH-R*-mRNA, non-radioactive ISH was performed during the
253 early luteal stage when its expression was at the highest relative mRNA levels. Specific
254 signals were targeted to luteal cells (Fig. 2B). No background staining was observed in
255 negative controls (sense probes) (Fig. 2C)

256

257 **Effects of deslorelin-treatment on luteal gene expression at mid-diestrus**

258 Whereas the expression of *STAR* did not differ significantly (P=0.09) between the two
259 groups (i.e. treated versus non-treated), the expression of *3βHSD* was higher (P=0.03)
260 in deslorelin-treated animals (Fig. 3). As for genes encoding for the PG system, the
261 expression of *COX2* (PTGS2) and *PTGES* was not affected by the treatment (P=0.6 and
262 P=0.2, respectively). Expression of the two PGE2 receptors was affected in CL of
263 treated dogs, showing opposite effects: *EP2* was significantly higher in treated animals

(P=0.04), while *EP4* expression was lower in that group (P=0.04) (Fig. 3). With regards to angiogenic factors of the *VEGF*-family, only the expression of *VEGFR1* was affected significantly (P=0.04) and was elevated in CL of deslorelin-treated animals, whereas *VEGFA* and *VEGRF2* did not differ (P=0.1 and P=0.6, respectively) (not shown).

Expression of the following genes did not differ between the two groups: *Kiss1-R* (P=0.3), *PRLR* (P=0.2), *PGR* (P=0.9), *ERα* (P=0.08) and *ERβ* (P=0.2).

In both deslorelin-treated and control dogs, the relative expression of *GnRH-R* and *Kiss1* was low and in some samples below the detection limits, preventing us from performing statistical analysis.

Discussion

In addition to its well-known properties in regulating the mammalian pituitary gland, and thereby, reproductive function, the GnRH-system exerts many paracrine, autocrine and endocrine actions in numerous extrapituitary tissues including cancer cells (Aguilar-Rojas and Huerta-Reyes 2009), prostate, ovary, (Cheung and Wong 2008), placenta (Schafer-Somi et al. 2015, Cheung and Wong 2008, Aguilar-Rojas and Huerta-Reyes 2009) and breast (Cheung and Wong 2008). While the role of this system in ovarian steroidogenesis (Sridaran et al. 1999a, Sridaran et al. 1999b) and apoptosis (Papadopoulos et al. 1999, Sridaran et al. 1998, Sridaran et al. 1999b, Sridaran et al. 1999a) has been demonstrated in several mammalian species, there is still a lack of information regarding its possible functions in the domestic canine species. Therefore, here, first we attempted to show the basic capability of canine CL to respond directly to GnRH, by investigating the mRNA expression of its receptor *GnRH-R* during the non-pregnant cycle (days 5, 15, 25, 35, 45 and 65 post-ovulation).

288 Indeed, although generally low, the transcripts encoding for *GnRH-R* were detectable in
289 luteal samples throughout the non-pregnant cycle. There was a gradual decrease
290 towards the mid-luteal phase and further downregulation was seen at the late luteal
291 stage. A similar expression pattern of *GnRH-R* was also described for the pregnant rat
292 ovary, where progression of the luteal phase was associated with diminishing
293 expression of immunoreactive GnRH-R (Sengupta et al. 2008). As detected by ISH,
294 *GnRH-R* mRNA was localized in luteal cells, matching similar expression pattern of
295 GnRH-R in other mammalian species, *e.g.*, monkey (Chakrabarti et al. 2008) and rabbit
296 (Zerani et al. 2010). Therefore, our findings imply possible regulatory involvement of
297 GnRH within the canine CL. This is supported by the presence of detectable amounts of
298 *Kiss1-R* in canine CL. The relative importance of locally-expressed GnRH-R for canine
299 luteal physiology remains, however, to be investigated further.
300 Hormonal approaches to postpone or prevent the onset of cyclicity in dogs are being
301 explored. In contrast to its natural pulsatile hypothalamic secretion, continuous
302 exogenous administration of a GnRH agonist results in down-regulation of the GnRH
303 receptors and desensitization of the pituitary gonadotrophs (Finch et al. 2009).
304 Furthermore, suppression of LH and FSH levels and gonadal steroidogenic activity is
305 observed, thereby blocking reproductive function (Rubion et al. 2006, Trigg et al.
306 2001). Therefore, in recent years, GnRH-analogs have been developed as a non-surgical
307 method to control reproduction in dogs, as well as in cats (Kutzler and Wood 2006,
308 Trigg et al. 2001).
309 The knowledge about the application of GnRH agonists in females is, however, still not
310 fully established, mostly due to the “flare-up” effect they may cause on the pituitary-
311 gonadal axis. In a previous study utilizing the same animals (Kaya et al. 2015), the

312 effects of deslorelin implants on epiphyseal closure, body development and time to
313 onset of puberty were investigated. It has been clearly demonstrated that deslorelin
314 could be used efficiently for delaying puberty in pre-pubertal dogs. Following this line,
315 in the present study, the possible long-term effects of Suprelorin treatment on canine
316 luteal function were assessed by investigating the expression of several factors involved
317 in establishment and maintenance of canine CL following the first estrus after long-term
318 prepubertal treatment. Accordingly, during the early luteal phase, PGs appear to be
319 among the most important factors involved in establishment of luteal function in the
320 dog (Kowalewski et al. 2013). Afterwards, during luteal maintenance, PRL acts as the
321 main luteotropic factor (Okkens et al. 1990), with its receptor, PRLR, being upregulated
322 at early- and mid-luteal phase (Kowalewski et al. 2011b). STAR protein is a key factor
323 regulating the provision of steroidogenic substrates and is one of the main downstream
324 responders in the steroidogenic cascade, the first enzymatic step of which is catalyzed
325 by 3βHSD. The expression of both proteins mirrors circulating P4 levels in the dog
326 (Kowalewski et al. 2006a, Kowalewski and Hoffmann 2008). Furthermore,
327 establishment of luteal function depends on vascularization, with the VEGF system
328 being the most prominent representative of the entire family of angiogenic and
329 vasoactive factors. This led us to investigate the expression levels of all these important
330 factors in CL from GnRH-treated dogs, enquiring about possible carry-over effects in
331 the following cycle after the postponed onset of estrus. Whereas *COX2* and *PTGES*
332 remained unaffected, the expression profiles of *EP2* and *EP4* receptors were changed
333 significantly: whereas *EP2* was elevated, *EP4* expression was lower in the CL of treated
334 animals. As for *EP2*, we found increased expression of one of the VEGF receptors,
335 *VEGFR1*. Interestingly, in contrast to unaffected *STAR* mRNA levels, we found

upregulated levels of *3βHSD* in treated animals. However, neither the enhanced expression of *EP2* and *VEGFR1* nor the elevated levels of *3βHSD* were related to increased luteal functionality, as similar P4 and E2 plasma levels were detected in both groups, *i.e.*, control and treated bitches. We interpreted this as a part of a possible feedback mechanism following suppression of pituitary function prior to ovulation and development of the luteal structure. The expression of *PRLR* and steroid hormone receptors, *PGR*, *ERα* and *ERβ*, remained unaffected.

In conclusion, by showing higher transcript abundance at the beginning of the luteal phase, the luteal presence of *GnRH-R* implies possible direct endocrine regulatory involvement of GnRH within the canine CL. Furthermore, despite an imbalance in the expression of some important luteotropic regulatory factors, the long-term delay of puberty by means of GnRH analog deslorelin treatment seems to have no negative carry-over-effects on subsequent reproductive activity and ovarian functionality in bitches. Certainly, due to the relatively low numbers of animals used in our study, the results presented herein are not definitive and future studies involving larger numbers of dogs should be undertaken. Nevertheless, here, the first insights into the local effects of GnRH in canine CL have been obtained, and the expression of *GnRH-R* has been shown for the first time in the dog CL.

Acknowledgements

Authors are grateful to Dr. Barry Bavister for careful editing of the manuscript. The technical expertise and contributions of Elisabeth Högger are greatly appreciated. Part

of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich. This research was supported partly by The Scientific and Technological Research Council of Turkey (TUBITAK-BİDEB 2219-1059B191401509) and by the Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich.

Conflict of interest

None of the authors has any conflict of interest to declare.

Authors' contributions

DK and AG contributed equally to this work and coordinated the respective parts of the project conducted at both of the universities, *i.e.*, The University of Kafkas, Kars, Turkey, where the animal experiments were performed, and at The University of Zurich, Zurich, Switzerland, where the laboratory part of the project was done. KD, GA, Sch-SS, MPK, AB and SA contributed to experimental design, critical discussion and interpretation of the data. Surgeries and tissue collections were conducted by KD and KM. Tissue processing and laboratory parts of the project, and writing of the manuscript, were performed by DK, AG, and MPK. All the authors read and approved the manuscript.

Figure Legends

Fig. 1. Distributions of progesterone (P4) and estradiol-17β (E2) concentrations (ng/ml and pg/ml, respectively) in control and deslorelin-treated bitches on the day of tissue collection ($P>0.05$) in Experiment 2.

Fig. 2. Expression of *GnRH receptor* (*GnRH-R*) (A) and localization of *GnRH-R* mRNA in canine CL (B) (Experiment 1). (A) Relative changes in GnRH-R mRNA expression were determined by real time time (TaqMan) PCR in luteal samples throughout diestrus in non-pregnant dogs. Bars with different letters differ significantly: d5 *vs*. d65 $P<0.001$, and d15 *vs*. d65: $P<0.01$. d5 - d65 = days after ovulation. (B) Localization of *GnRH-R* mRNA as determined by *in situ* hybridization (ISH) in canine CL during early diestrus; representative microphotographs from d15 after ovulation are shown. Signals were localized to luteal cells (solid arrowheads). (C) There is no background staining in negative controls.

Fig. 3. Expression of selected target gene mRNAs as determined by real time (TaqMan) PCR in luteal samples collected from deslorelin-treated and control dogs following the first estrus. Numerical data are presented as geometric means (Xg) ± geometric standard deviation (SD).

Table 1. List of primers and TaqMan probes used for the semi-quantitative PCR

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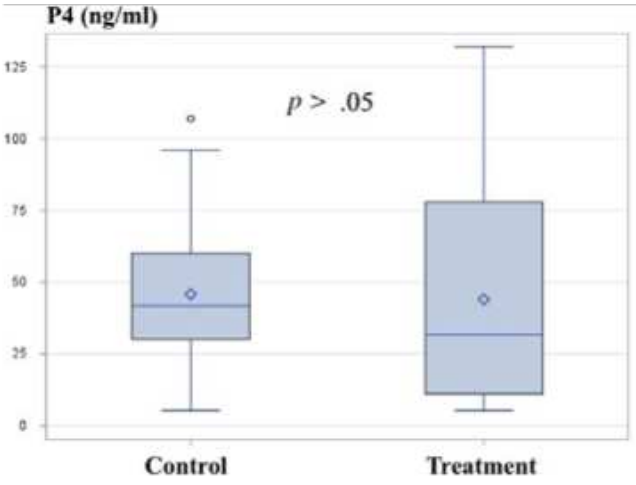
Gene	Primer sequence	Product length (bp)	Accession number
Kiss 1	For: 5'-CCT GGT TTC TTG GCA GCT AAT G -3' Rev: 5' -GTC TCC ATG GGT GCC ACC TT-3' TaqMan: 5' –TCT CCT CTG TGC CAC TTC CTT CAG GGAG-3'	81	KJ512885
GAPDH	For: 5'-GCT GCC AAA TAT GAC GAC ATC-3' Rev: 5'-GTA GCC CAG GAT GCC TTT GAG-3' TaqMan p: 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'	75	AB028142
3β-HSD	For: 5-GGGTACTCAGCTCCTGTTGGAA-3' Rev: 5-GCCACCTCTATGGTGCTGGTAT-3' TaqMan p: 5-TGCCCAGGCTAGTGTGCCGATCTT-3'	78	AY739720.1
PGR	For: 5'-CGA GTC ATT ACC TCA GAA GAT TTG TTT-3' Rev: 5'-CTT CCA TTG CCC TTT TAA AGA AGA-3' TaqMan: 5'-AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3'	113	NM_001003074
StAR	For: 5'-CGA GGC TCC ACC TGT GTG T-3' Rev: 5'-CCT TTC TGC TCA GGC ATC TC-3' TaqMan: 5'-CTG GCA TGG CCA CAC ATT TC-3'	65	EF522840
COX2	For: 5'-GGA GCA TAA CAG AGT GTG TGA Rev: TGT G-3' 5'-AAG TAT TAG CCT GCT CGT CTG GAA T-3' TaqMan: 5'-CGC TCA TCA TCC CAT TCT GGG TGC-3'	87	HQ110882
PTGES	For: 5'-GTC CTG GCG CTG GTG AGT-3' Rev: 5'-ATG ACA GCC ACC ACG TAC ATC T-3' TaqMan: 5'-TCC CAG CCT TCC TGC TCT GCA GC-3'	89	NM_001122854
VEGFA	For: 5'-GTG CCC ACT GAG GAG TTC AAC-3' Rev: 5'-CCC TAT GTG CTG GCC TTG AT-3' TaqMan: 5'-CAC CAT GCA GAT TAT GCG GAT CAA ACC-3'	72	NM001003175
VEGFR 1	For: 5'-TGC CTG AAA CAG TGA GAA AGG A-3' Rev: 5'-TGC AGA ACT GTT TGC CAT TCC-3' TaqMan: 5'-AAA GGC TGA GCA TTA CTA AAT CTG CCT-3'	81	AF262963
VEGFR 2	For: 5'-TGA CAT GGC CTC GGT CAT T-3' Rev: 5'-TGT TGG TCG CTA ACA GAA GCA-3' TaqMan: 5'-CTA CGT TCA AGA TTA CAG GTC TCC ATT-3'	75	DQ269018/ NM001048024
EP 2	For: 5'-CAC CCT GCT GCT GCT TCT C-3' Rev: 5' -CGG TGC ATG CGG ATG AG-3' TaqMan: 5'-TGC TCG CCT GCA ACT TTC AGC GTC-3'	78	AF075602
EP 4	For: 5'-AAA TCA GCA AAA ACC CAG ACT TG-3' Rev: 5'-GCA CGG TCT TCC GCA GAA-3' TaqMan: 5'-ATC CGA ATT GCT GCT GTG AAC CCT ATC C-3'	96	AF177934
ERα	Forward: 5'-CCC ATG GAG GAG ACA AAC CA-3' Reverse: 5'-CCC TGC CTC GGT GAT ATA-3' TaqMan : 5'-CAC GGG CCC AAC TTC ATC ACA TTC C-3'	93	XM533454
ERβ	Forward: 5'-CCC AGC CCC TTC A-3' Reverse: 5'-AAT CAT ATG CAC GAG TTC CTT GTC-3' TaqMan : 5'-CCT CCA TGA TGA TGT CCC TGA CC-3'	78	XM861041
PRLR	Forward: 5'-GGA TCT TTG CCG TTC TTT-3' Reverse: 5'-AAG GAT GCA GGT CAC CAT GCT AT-3' TaqMan : 5'-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3'	92	HQ267784

Table 1

A


P4 (ng/ml)
p > .05
125
100
75
50
25
0
Control
Treatment

B

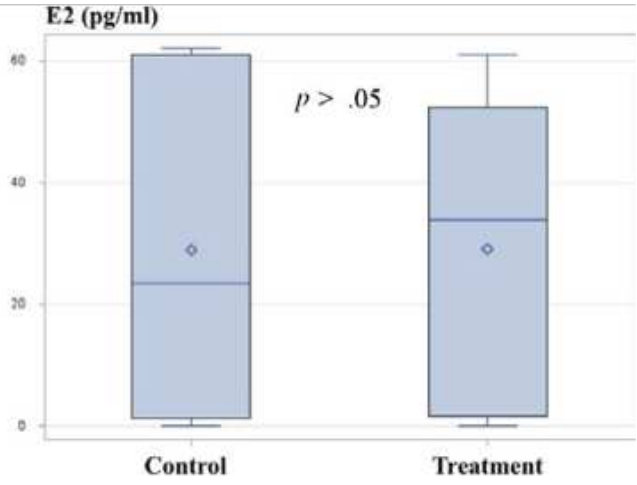
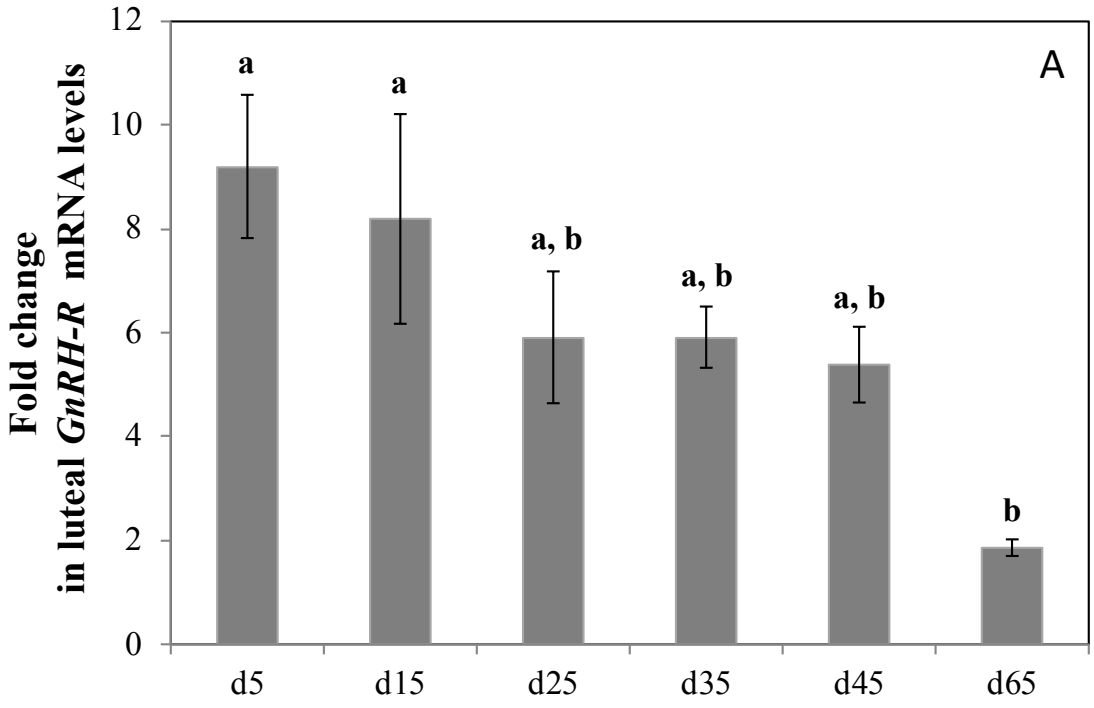

E2 (pg/ml)
p > .05
60
40
20
0
Control
Treatment

Figure 1


A
Fold change in luteal GnRH-R mRNA levels
12
10
8
6
4
2
0
a
a
a, b
a, b
a, b
b
d5
d15
d25
d35
d45
d65

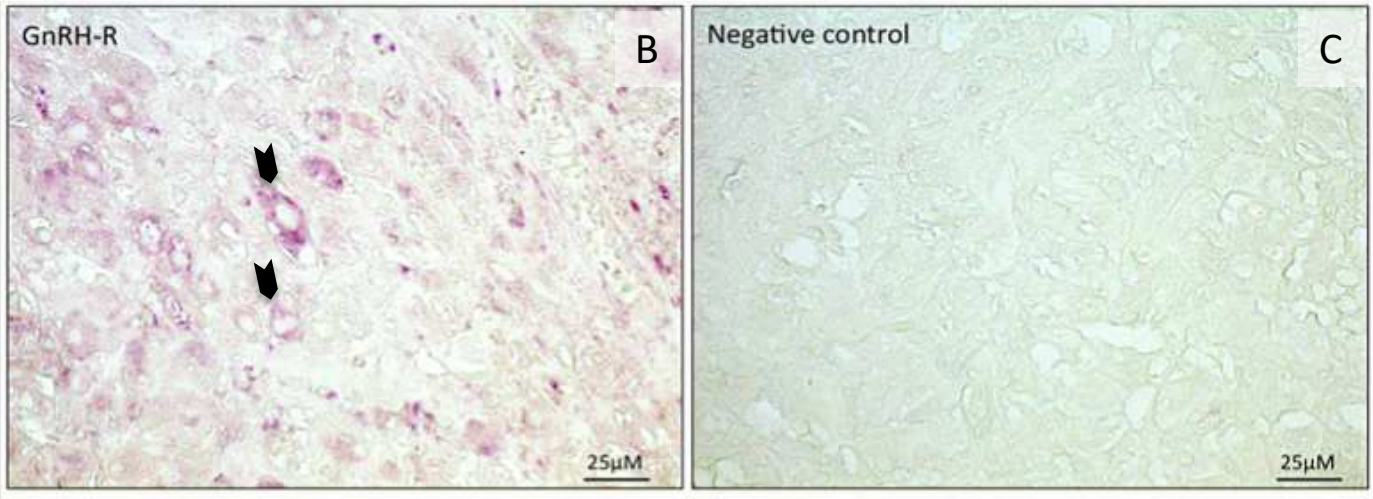

GnRH-R
B
25µM
Negative control
C
25µM

Figure 2

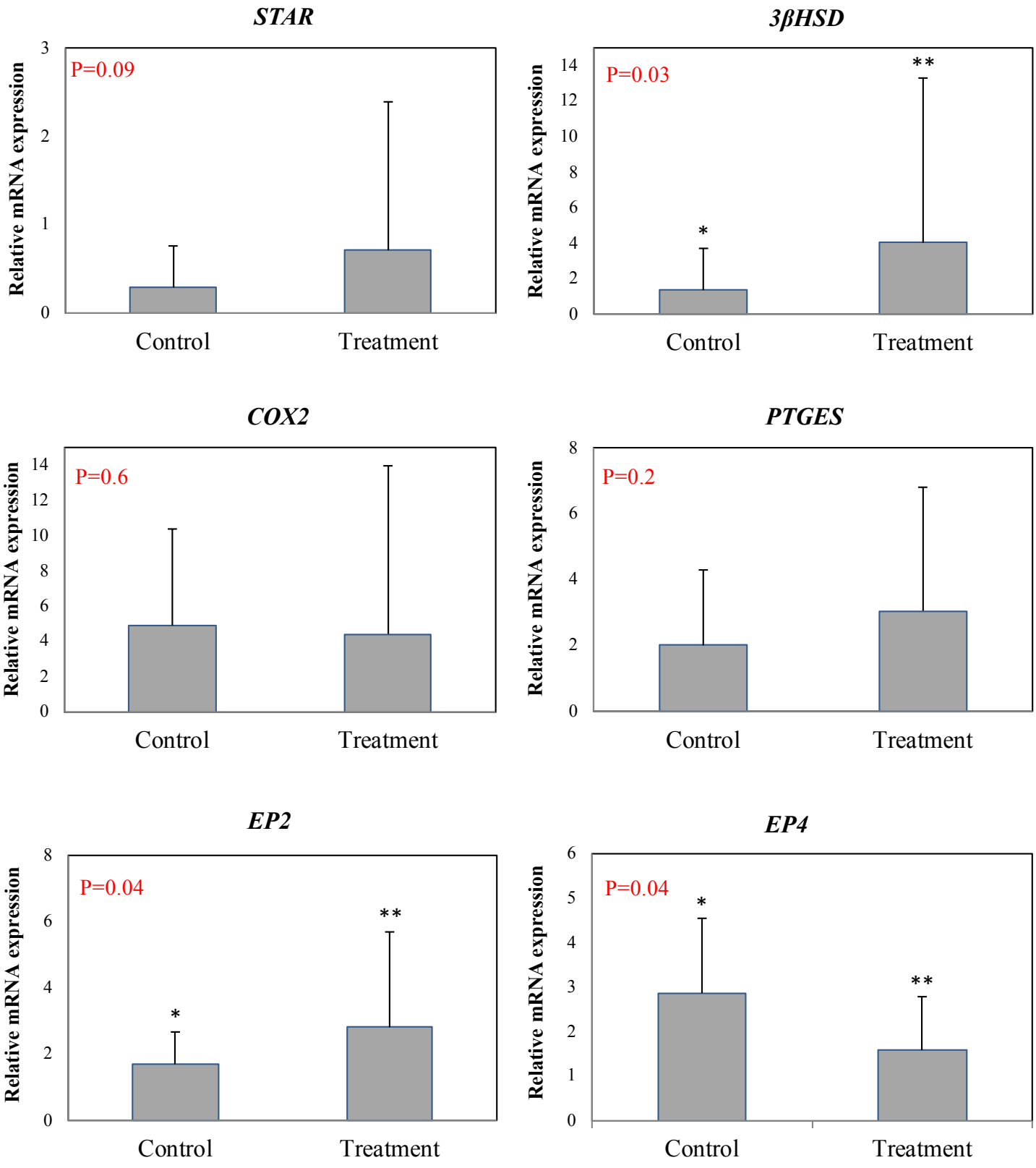

STAR
P=0.09
Relative mRNA expression
0
1
2
3
Control
Treatment
3βHSD
P=0.03
Relative mRNA expression
0
2
4
6
8
10
12
14
*
**
Control
Treatment
COX2
P=0.6
Relative mRNA expression
0
2
4
6
8
10
12
14
Control
Treatment
PTGES
P=0.2
Relative mRNA expression
0
2
4
6
8
Control
Treatment
EP2
P=0.04
Relative mRNA expression
0
2
4
6
8
*
**
Control
Treatment
EP4
P=0.04
Relative mRNA expression
0
1
2
3
4
5
6
*
**
Control
Treatment

Figure 3